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Anaerobic Biodegradation of Nitrogen-Substituted and Sulfonated Benzene Aquifer Contaminants

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Anaerobic Biodegradation of Nitrogen-Substituted and Sulfonated Benzene Aquifer Contaminants

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ABSTRACT

A literature survey of ground water contaminants indicated that aquifers are repositories for hazardous wastes, including N- and S-substituted benzene derivatives. We therefore examined the susceptibility of several anilines, benzamides, benenesulfonic acids and benenesulfonamides to anaerobic metabolism by aquifer microorganisms. Under sulfate-reducing and methanogenic conditions the carboxylated anilines were biotransformed within 1 to 3 months while unsubstituted or methylated anilines required longer incubation times. Benzamide as well as an aryl methyl and an N-methyl derivative were biodegraded under both redox conditions. The anaerobic degradation of the N-methylated benzamide was favored in sulfate-reducing rather than methanogenic incubations. However, the addition of a second N-alkyl group rendered the resulting compounds resistant to anaerobic decay. Only 1 of 7 benzenesulfonates and 2 of 5 benzenesulfonamides proved amenable to anaerobic metabolism. We found 37-75% of the theoretically expected amount of methane from aquifer slurries amended with the aminobenzoic acids, benzamide, and p-toluamide. In the sulfate-reducing aquifer slurries 89-100% of the oxidized benzamide, p-toluamide and N-methylbenzamide could be accounted for by sulfate reduction. These results help indicate which hazardous waste constituents will likely persist in anoxic aquifers and the types of chemical substitution patterns that favor anaerobic biotransformation.

INTRODUCTION

Various reports of ground water quality indicate that substituted anilines, nitrobenzenes, benzenesulfonamides and benzamides are frequent aquifer contaminants (Table 1). Methylated or higher alkylated derivatives of these compounds are particularly important pollutants. Amino substituted aromatic compounds are synthetic organic chemicals widely used for the manufacture of dyestuffs, pigments, rubber products, and agricultural chemicals (1). In 1987, U.S. production of aniline alone exceeded 900 million pounds (2). Aniline and methylated anilines (toluidines) could also be detected in the waste water from coal gasification sites (Table 1). In addition, such chemicals can also be formed during the catabolism of naturally-occurring compounds. Thus, aminobenzoic acid has been established as an anaerobic degradation product of the amino acid tryptophan (3).

TABLE 1

Detection of Nitrogen-Substituted Aromatic Compounds, Benzenesulfonamides and Benzeneamides in Ground Water

Chemical group/ individual compounds	Use or likely origin of pollutant	Location	Reference
alkylbenzenesulfonamides e.g. N-ethyl-p-toluenesulfonamide, N-ethyl-o-toluenesulfonamide p-toluenesulfonamide	plasticizers, fungicide, synthesis of resins, mildewcide in paints	aquifer underlying a municipal landfill, Norman, OK	50 51
alkylbenzamides e.g. N,N-diethylbenzamide, N-isobutyl-4-methylbenzamide alkylbenzenesulfonamides e.g. N,N,4-trimethylbenzenesulfonamide	leachate	aquifer underlying a municipal landfill, Norman, OK	G.Smith *
alkylbenzenesulfonamides e.g. N-ethyl-p-toluenesulfonamide, N-butylbenzenesulfonamide	leachate	sanitary landfill Barcelona, Spain	52
m-nitrophenol aniline	creosote waste, manufacture of dyes, resins, perfumes, medicinals, solvents	Waste site, Conroe, TX	53
chloroanilines e.g. 3,4-dichloroaniline	solvents, manufacture of dyes, pesticides, explosives, medicinals	River Rhein and ground water infiltrate, Netherlands	54
p-chloronitrobenzene	dye chemistry		
nitrobenzene	manufacture of aniline and pyroxyline compounds, in shoe polishes		
nitrotoluene	manufacture of dyes, toluidines, nitrotoluic acids		
alkylanilines, e.g. toluidine, N-ethylaniline N-4-butylbenzenesulfonamide	manufacture of dyes and other organic chemicals		
aniline o-, m-, p-toluidine	pollutants from coal gasification process	near underground gasification site, Wyoming	55
chloroanilines chlorotoluidines chloronitrobenzenes nitrotoluenes	Rhine river water and the dune infiltrated ground water	Wijk aan Zee, dune infiltration area, Netherlands	56

* personal communication

Similarly, nitroaromatic chemicals are frequently used as pesticides, drugs, dyes, and explosives. They are known waste products of several industries and also arise as the breakdown products of several herbicides (4). Microorganisms can reduce aryl nitro groups to aniline derivatives as demonstrated under both aerobic (5,6) and anaerobic conditions (7).

Azo dyes are colorants used in the textile, food and cosmetic industry and U. S. production of this group of chemicals exceeds 220 million pounds per year (8). These chemicals are not easily biodegraded in aerobic waste-water treatment systems. However, under anaerobic conditions, azo dyes can be biotically (9) and abiotically reduced to aromatic amines (10). Azo dyes are of environmental concern since their breakdown products can be carcinogens (11,12).

Aromatic sulfonic acids are used as wetting agents, dispersants and as intermediates in the synthesis of azo dyes, (13). During the acidic sulfite process, lignin is converted to sulfonated aromatic derivatives which are more resistant to microbial attack relative to lignin itself (14). Lignosulfonates can be major contaminants in waste water from paper manufacturing plants (14). Such compounds are generally sulfonated at the alkyl or alkene substituents (14). The branched alkyl(C9 or C12)benzene sulfonates are anionic surfactants which are highly recalcitrant chemicals (15). Initial transformation of these substances during sewage treatment results in i) the formation of nonylphenolmono- and diethoxylates released in effluents and ii) the accumulation of the more toxic nonylphenol in digested sludge (up to 2.6 mmol/kg) (16). The more readily degradable linear akylbenzene sulfonates replaced the branched chain surfactants and represent a U.S. market of 400,000 tons per year (15).

The aerobic microbial transformation of benzenesulfonic acids has been found (13,17), but the arene-sulfonic acid bond confers real xenobiotic character to this class of compounds (17). The anaerobic removal of the aryl sulfone substituent has not yet been observed (15). To our knowledge, the anaerobic metabolic fate of benzenesulfonamides and benzamides has never been reported. Similarly, the aerobic biodegradation of anilines is reasonably well characterized (18-20), but the fate of such compounds in the absence of molecular oxygen is largely unknown (21).

The goal of our study was to test the susceptibility of a variety of N- and S-substituted aromatic compounds to anaerobic biodegradation by aquifer microorganisms. The test structures included several substituted anilines, benzeneamides, benzenesulfonic acids and benzenesulfonamides. The model compounds were tested in aquifer slurries held under both methanogenic and sulfate reducing conditions. We found that most of the nitrogen-substituted benzenes were biotransformed or mineralized while the sulfonated benzenes tended to persist under these incubation conditions. Our studies also suggest that carboxylated anilines are more readily biodegraded than aniline or methylated anilines. In general, benzamides were biodegradable, but alkyl substitution at the nitrogen atom rendered the chemical derivatives more persistent. A similar effect was observed for the benzenesulfonamides under methanogenic conditions.

MATERIALS AND METHODS

Chemicals

All chemicals used were analytical grade or purchased at the highest available purity. Aldrich Chemical Co. (Milwaukee, WI) supplied aniline, o-, m-, p-toluidine, m-aminobenzoic acid, benzamide, N-methylbenzamide, N,N-dimethylbenzamide, p-toluamide, N,N-diethyl-m-toluamide, benzenesulfonamide, p-toluenesulfonamide, sulfanilic acid, p-toluenesulfonic acid. We purchased N-methylbenzenesulfonamide, N,N-diethyl-p-toluenesulfonamide, N-ethyl-toluenesulfonamide (a mixture of o- and p- isomers), benzenesulfonic acid, orthanilic acid, and metanilic acid from Tokyo Chemical Industry Co. , Ltd. (Tokyo, Japan). p-Phenolsulfonic acid, and benzosulfonic acid (p-sulfobenzoic acid) were obtained from Chemical Services, Inc. (West Chester, PA) while Calbiochem Chemical Co. (Los Angeles, CA) and Nutritional Biochemical Co. (Cleveland, OH) supplied o-aminobenzoic acid (anthranilic acid) and p-aminobenzoic acid, respectively.

Sampling and Incubation of Aquifer Slurries

Aquifer material and site water was sampled from two well characterized

sites adjacent to the Norman municipal landfill as previously described (22). The sampling was conducted in late spring. The water table at the methanogenic site was 1.35 m below the surface, had a temperature of 18°C, a pH of 6.8, a total organic carbon content (TOC) of 288 mg/L, and a sulfate concentration of less than 0.1 mM. The water table at the sulfate reducing site was 1.15 m below the surface, had a temperature of 16°C, a pH of 7.2, the TOC was 14.4 mg/L and a sulfate concentration 2.1 mM. Aquifer samples were transported to the laboratory and placed in an anaerobic glove box where 50 ± 1 g aquifer material and 50 ml site water were aseptically transferred to sterile glass serum bottles (160 ml). The bottles were closed under an atmosphere of N_2/CO_2 (80/20) and reducing conditions were controlled by the addition of 1 mM Na_2S. Filter sterilized resazurin (0.0002%) was added as a redox indicator. The bottles were sealed with 1 cm thick butyl rubber stoppers and crimped with aluminum seals. The bottles were stored at least 5 days prior to the addition of the aromatic test compounds. At the start of experiments with the benzamides and benzenesulfonamides, the initial sulfate concentration was adjusted to 7.5 mM in the SR aquifer slurries.

Anaerobic and filter sterilized (0.2 µm) stock solutions of all aromatic test chemicals (10 mM benzamides and benzenesulfonamides, 60 mM aniline, aminobenzoic acid, toluidine) were prepared in distilled water. The more hydrophobic N,N-diethyl-p-toluenesulfonamide was dissolved in pure methanol to a concentration of 1.2 M. All aromatic model compounds were aseptically added to aquifer slurries to reach an initial concentration of about 0.2 mM. The experiments were conducted in duplicate and autoclaved aquifer slurries (121°C, 15 lb/in^2, 180 min) served as controls. The biodegradation of each substrate was tested individually. The bottles were incubated in the dark at room temperature. One ml samples of the aquifer slurries were removed by syringe immediately after the addition of the substrates and periodically thereafter during the course of the experiment. The samples were stored at -10°C until analyzed.

Substrate and Metabolite Analysis

The stored subsamples from aquifer slurries were thawed and centrifuged (5 min, 8000 x g) prior to reversed phase HPLC analysis with a C18 column. The hardware used for this analysis has been previously described (23,24). A mixture of acetonitrile and acetate buffer (50 mM, pH 4.5) was used as the mobile phase at a flow rate of 1.5 ml/min. Compound detection was by UV absorbance with a variable-wavelength spectrophotometer (Beckman model 165). The analytical conditions (acetonitrile/buffer ratio, wavelength in nm, retention time in min) for each compound are given below: aniline (7/8, 280, 4.05), o-toluidine (7/8, 280, 5.25), m-toluidine (7/8, 280, 5.20), p-toluidine (7/8, 280, 5.05), o-aminobenzoic acid (4/11, 310, 4.60), m-aminobenzoic acid (1/14, 295, 8.55), p-aminobenzoic acid, 4/11, 265, 3.10), benzamide (5/10, 270, 3.05), N-methylbenzamide (6/9, 270, 3.12), N,N-dimethylbenzamide (5/10, 270, 4.63), p-toluamide (5/10, 270, 3.95), N, N-diethyl-m-toluamide (10/5, 270, 3.50), benzenesulfonamide (5/10, 270, 3.40), N-methylbenzenesulfonamide (8/7, 270, 3.04), p-toluenesulfonamide (7/8, 270, 3.16), N,N-diethyl-p-toluenesulfonamide (10/5, 270, 4.35), N-ethyl-p/o-toluenesulfonamide (10/5, 270, 3.00). The mobile phase for the analysis of the substituted benzenesulfonic acids was a 100 mM-potassium phosphate buffer, pH 7.0 (25), and the analytical conditions (flow rate in ml/min, wavelength in nm, retention time in min) were as follows: benzenesulfonic acids (1.5, 264, 8.20), orthanilic acid (1.5, 296, 5,05), metanilic acid (1.5, 296, 3.09), sulfanilic acid (1.2, 245, 2.83), p-toluenesulfonic acid (1.4, 260, 18.2), p-phenolsulfonic acid (1.4, 275, 2.71) and p-benzosulfonic acid (1.4, 275, 1.85). The injection loop size was 100 µl and the detection limit for all substrates was 10 µM or less.

Methane and Sulfate Analysis

The concentration of methane was determined by flame ionization gas chromatography as previously described (26) or with a Varian model 3300 gas chromatograph using a 1.8 m x 0.32 cm stainless steel column packed with 80/100 mesh poropack Q. Nitrogen was used as a carrier gas at a flow of 30 ml/min. The temperature of the injector, column, and detector was 100°C, 105°C, and 120°C, respectively. External standards were employed and kept in stoppered

glass serum bottles. Concentrations of methane in the headspace of methanogenic incubations were typically on the order of a few percent.

The aqueous phase of the aquifer slurries were sampled for sulfate analysis, centrifuged at 8000 x g for 3 min, and analyzed by anion exchange HPLC. The mobile phase (4.0 mM potassium hydrogen phthalate, pH 5.0) was pumped at 2.5 ml/min with a LDC/Milton Roy minipump (model 396) through an anion exchange column (Vydac, 250 x 4.6 mm, Alltech Associates, Inc., Deerfield, IL). Sulfate exhibited a retention time of 6.3 min and was detected with a Wescan (Santa Clara, CA) conductivity detector (model 213-505). The detection limit was at least 0.1 mM.

Stoichiometry of Methane Formation

We applied the general Buswell equation (27) in order to calculate the stoichiometry of biotransformation of the substrates to methane:

$$C_nH_aO_bN_cS_d + (n - a/4 - b/2 + 7c/4 + d/2)\ H_2O \longrightarrow (n/2 - a/8 + b/4 - 5c/8 + d/4)\ CO_2 + (n/2 + a/8 - b/4 - 3c/8 - d/4)\ CH_4 + cNH_4HCO_3 + dH_2S.$$

For the calculations involving aromatic acids the protonated form was assumed. The number of moles of CH_4 expected per mol of substrate is 3.5 for aminobenzoic acid, 3.75 for benzamide and 4.5 for p-toluamide.

The disappearance of the model compounds was measured by HPLC to calculate the µmoles of substrate depleted in the serum bottles (60 ml liquid volume). This value was then multiplied by the expected CH_4 stoichiometry to obtain the expected amount of CH_4 (µmoles) in the aquifer slurry due to the mineralization of the parent compound. We then accounted for the distribution of methane between the headspace and liquid phases of the serum bottles (Henry coefficient = 27.2; ref 28) and the volume of gas and liquid (84 ml/60 ml). We calculate that 97.4% of the methane partitioned to the headspace of the serum bottles. The total amount of methane formed in the serum bottles was corrected for the amount produced in substrate unamended controls. The percent CH_4 recovery is calculated from the amount of CH_4 measured divided by the amount of CH_4 expected.

RESULTS

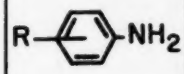
We tested the biodegradability of 24 model chemicals in sterile and non-sterile anaerobic aquifer slurries obtained from two sites within a shallow aquifer underlying the Norman municipal landfill. Sulfate reducing conditions (SRC) predominated at one aquifer site while methanogenesis (MC) prevailed in the other (22). The model substrates represented four different chemical groups including aminosubstituted benzenes (anilines), benzeneamides, benzenesulfonic acids, and benzenesulfonamides. Previous studies of the ground water quality showed a variety of contaminants including benzamides and benzenesulfonamides in the aquifer (Table 1).

Biotransformation of Aminosubstituted Aromatic Compounds.

Table 2 summarizes our findings on the fate of amino substituted aromatic chemicals. No abiotic loss was observed for aniline or the aminobenzoic acids. However, about 27-35% of the toluidine isomers disappeared in sterile incubations monitored over a 10 months period. The data given in Table 2 are corrected for the abiotic loss and thus indicate only a biotic effect. In general, aniline and the three toluidine isomers were recalcitrant under both redox conditions. No biotic transformation of these substrates could be detected in aquifer slurries incubated under MC. However, partial metabolism occurred in the samples from the SR site after an adaptation period of several months. Within ten months, 39% of the aniline amendment and 73% of m-toluidine was biodegraded in the SR aquifer slurries. Nevertheless, no significant transformation of the o- or p-toluidine could be detected over the same time period. We estimate a first-order decay rate of 0.12 $month^{-1}$ for aniline and 0.65 $month^{-1}$ for m-toluidine during the last two months of incubation.

Contrary to aniline and the toluidine isomers, aminobenzoic acids were

TABLE 2
Microbial Degradation of Aminosubstituted benzenes in aquifer slurries from a sulfate reducing (SR) and a methanogenic (M) site

 Aniline	(R) group	Substrate Concentration (μ M)									
		SR slurries					M slurries				
		0mth	1mth	3mth	8mth	10mth	0mth	1mth	3mth	8mth	10mth
Aniline	H	203	218	200	155	123	198	214	195	227	196
o-Toluidine [a]	CH3	196	186	225	204	194	210	212	224	215	230
m-Toluidine [a]	CH3	206	181	228	205	56	194	173	203	232	210
p-Toluidine [a]	CH3	183	191	213	201	236	176	157	181	146	188
o-Aminobenzoate	COOH	203	122	128	<2		205	176	16	<2	
m-Aminobenzoate	COOH	200	188	2	<2		172	140	11	<2	
p-Aminobenzoate	COOH	197	173	16	<2		192	182	61	<2	

[a]The data are corrected for abiotic loss of the substrate from sterile controls assuming first-order rate elimination

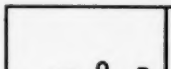
biodegraded within 3 months under both redox conditions and no parent substrate could be detected after 8 months (Table 2). For the most part, the rate of substrate disappearance was similar for all isomers under both redox conditions. Typically, an adaptation period of 1 month was followed by a rapid substrate loss between the first and third month. We estimated first-order decay rates for o-, m- and p-aminobenzoic acid over this time period and found them to be similar. In contrast to the other isomers, o-aminobenzoic acid was not transformed in this time period under SRC and the maximum decomposition rate was observed between the third and the eighth month. Under SRC we found maximum rates of 0.83, 2.3 and 1.2 $month^{-1}$ for the removal of o-, m-, and p-aminobenzoic acid respectively, whereas under MC the rates were 1.2, 1.3 and 0.6 $month^{-1}$, respectively. Our search for potential metabolic intermediates of the aminobenzoic acids included assays for benzoate, o-, m-, p-hydroxybenzoic acid, phenol and aniline, but none of these compounds were found. However, all three isomers were at least partially mineralized to methane in aquifer slurries obtained from the M site. The degradation of ortho- (205 μM), meta- (172 μM), and para-aminobenzoic acid (192 μM) resulted in the formation of 17.3, 15.0, and 14.6 μmoles methane by the eighth month. This accounts for 40.5, 42.0, and 36.6% of the theoretically expected amount of methane, respectively.

In non-sterile aquifer slurries from the SR site amended with the aminobenzoic acids, the concentration of sulfate dropped from an initial 1.75 mM to 0.1 mM by the end of the experiment. This must be compared to substrate unamended controls where sulfate levels dropped to 0.2 mM. Electron balance studies are not possible since the kinetics of sulfate reduction at this concentration is probably dependent on the level of sulfate (29) and methanogenesis might contribute in the catabolism of the substrates. However, we could not measure a significant increase of methane production in substrate amended over unamended SR aquifer slurries. The methane formation in the SR incubations was at least 5 times slower than comparable M incubations.

Biotransformation of Benzamides

We tested the anaerobic biodegradability of benzamide and four derivatives including arylmethylated and N-alkylated benzamides. A summary of our findings with these structures is shown in Table 3.
No abiotic substrate loss occurred in the sterile aquifer samples, so the amount of depletion in non-sterile samples can be considered microbially mediated. Within 1 month, >49% of benzamide and p-toluamide were removed and >99% of these substrates were transformed within 8 months in aquifer slurries from both sites. Interestingly, N-methylbenzamide was more readily transformed in the SR incubations (100%) than in the M incubations (16%) within 11 months. The double N-alkyl substituted compounds, N,N-dimethylbenzamide and

TABLE 3
Microbial Degradation of alkylated benzamides in aquifer slurries from a sulfate reducing (SR) and a methanogenic (M) site.

 Chemical	Substituents			Substrate Concentration (µ M)							
				SR slurries				M slurries			
	R1	R2	R3	0mth	1mth	8mth	11mth	0mth	1mth	8mth	11mth
Benzamide	H	H	H	261	144	1	0	157	94a	0	0
N-Methyl-benzamide	H	H	CH3	195	170	0	0	187	175	172	128a
N,N-dimethyl-benzamide	H	CH3	CH3	192	188	182	174	176	172	173	175
p-Toluamide	CH3	H	H	228	115	1	0	213	96	0	0
N,N-Diethyl-m-toluamide	CH3	C2H5	C2H5	171	194	198	199	194	192	190	199

[a] Only one non-sterile replicate showed substrate disappearance

N,N-diethyl-m-toluamide, showed no significant substrate loss over the same incubation time.

To examine the effect of substrate concentration on anaerobic biodegradation, benzamide was incubated at concentrations of 50, 200, 500 and 2000 µM in aquifer slurries obtained from both sites. Complete substrate loss was observed at all employed concentrations, except the 2000 µM experiment held under SRC. In M aquifer slurries amended with 500 µM and 2000 µM substrate, 83.5 µmoles and 136.5 µmoles methane were produced, respectively. This represents a recovery of 74.7 and 27.6%, respectively, of that theoretically expected. Presumably benzamide metabolites accumulated at the higher concentration. It is not clear whether the differences in mineralization are due to the differential kinetics of parent compound and metabolite decay or to the toxicity of these substances to the requisite microorganisms.

The initial sulfate concentration in the aquifer slurries from the SR site dropped from 7.5 mM to 5.8 mM in unamended samples and not lower than 3 mM in any of the incubation receiving benzamide or its derivatives. The substrate was completely depleted in the samples receiving 200 and 500 µM benzamide and partially degraded in that amended with 2000 µM (16.1%). Concomitant with substrate loss was a decrease in sulfate concentration to 0.3 mM, 1.6 mM and 2.8 mM in the substrate amended slurries over the unamended controls. Assuming sulfate reduction to sulfide, we calculate that 40.0%, 85.1% and 100.0%, respectively of the substrate mineralization can be coupled with sulfate reduction in the samples with 200, 500, and 2000 µM benzamide. In the SR aquifer slurries with 500 µM benzamide the degradation was coupled with the production of 20.8 µmole methane, whereas no additional methane formation could be measured relative to an unamended control at 2000 µM. The methane formed in the SR incubations receiving 500 µM benzamide accounts for 19.8% of the theoretically expected amount. Thus, at this substrate concentrations, at least a partial flow of carbon and energy through methanogenesis was possible. The fact that no methane was formed at 2000 µM benzamide and that the substrate was only partially removed might be a result of the toxicity of this substrate at high concentrations to the catalyzing microorganisms.

In addition to benzamide, p-toluamide degradation under MC also resulted in the formation of methane. The disappearance of 12.9 µmoles p-toluamide was accompanied by the formation of 37.3 µmoles methane or 64.2 % of the theoretical recovery. The partial biotransformation of N-methylbenzamide in the M aquifer slurries did not result in the formation of significant amounts of methane over unamended controls. Similarly, the amount of methane found in aquifer slurries from the SR site amended with 200 µM benzamide or toluamide was not different from the unamended controls.

The complete removal of p-toluamide and N-methylbenzamide (0.2 mM each) in the SR aquifer slurries was coupled with the reduction of 1.2 mM and 0.8 mM sulfate, respectively. This indicates that 133% and 88.9% respectively of these substrates could be mineralized under sulfate reducing conditions.

Biodegradability of Benzenesulfonic Acids and Benzenesulfonamides

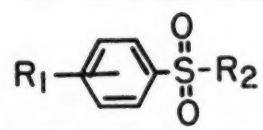
Of the model benzenesulfonic acid and derivatives, only one was degradable under anaerobic conditions. p-Benzosulfonic acid was biotransformed by the end of the experiment in the SR and M incubations, respectively (Table 4). However, no increased sulfate reduction or methane formation could observed in this sample compared to an unamended control aquifer slurries. No abiotic loss of the various substrates could be detected.

Similarly, benzenesulfonamide and its aryl or N-alkylsubstituted derivatives were also investigated for their fate in anoxic aquifer slurries (Table 4). Only two of the alkylsubstituted compounds were biotransformed under the test conditions. Benzenesulfonamide was tested in M aquifer slurries at concentrations ranging from 50 - 2000 µM. In one duplicate from the 500 µM test, biotic substrate loss (41.2%) was detected along with the accumulation of a more polar compound (shorter retention time by HPLC analysis). While not identified, the more polar compound was not benzenesulfonic acid which might have conceivably formed by amide hydrolysis.

In addition to benzenesulfonamide, about 20% of p-toluenesulfonamide disappeared from the M aquifer slurries. The small amount of depletion could not be correlated with methane production over background controls.

TABLE 4

Fate of Benzenesulfonic acids and benzenesulfonamides in aquifer slurries from a sulfate reducing (SR) and a methanogenic (M) site.

 Chemical Compound	Substituents R1	R2	Substrate Concentration (µ M) SR slurries 0 mth	SR slurries 13 mth	M slurries 0 mth	M slurries 13 mth
Benzenesulfonic Acid	H	O H	205	198	204	196
Orthanilic Acid	NH2 (ortho)	O H	195	198	199	197
Metanilic Acid	NH2 (meta)	O H	187	206	188	219
Sulfanilic Acid	NH2 (para)	O H	117	120	143	135
p-Toluenesulfonic Acid	CH3	O H	173	181	171	158
p-Phenolsulfonic Acid	O H	O H	188	198	194	235
p-Benzosulfonic Acid	C O O H	O H	199	0	187	98
			0 mth	10 mth	0 mth	10 mth
Benzenesulfoneamide	H	NH2	201	201	454	267[a]
p-Toluenesulfoneamide	CH3	NH2	207	233	227	177[b]
N-Methylbenzenesulfoneamide	H	NHCH3	203	210	177	212
N,N-Diethyl-p-toluenesulfoneamide	CH3	N(C2H5)2	147	149	128	133
N-Ethyl-p/m-toluenesulfoneamide	CH3	NH(C2H5)	199	208	194	221

[a] Only one non-sterile replicate showed substrate disappearance. Benzenesulfonamide was tested in aquifer slurries at four concentrations (50, 200, 500 and 2000 µM). [b] The value is corrected for the abiotic loss of the substrate from sterile controls.

DISCUSSION

A total of 24 model compounds representing four different groups of chemicals were tested for their biodegradability in aquifer slurries from a SR and a M site. The nitrogen-substituted compounds included anilines and benzamides while the sulfonated benzenes include sulfonic acids and sulfonamides.

Aminosubstituted benzenes were relatively easily biodegraded when the aromatic nucleus was substituted with a carboxy group (Table 2). This is in contrast to aniline and the methylated anilines (toluidines) which proved

generally recalcitrant. Additionally, methane formation from all three aminobenzoic acids could be demonstrated in incubations from the M site, but no significant methane accumulation over unamended controls was measured when either aniline or any of the methylated anilines were incubated in the same type of aquifer slurries. The biodegradability of this group of compounds might be somewhat better under SRC since aniline and m-toluidine were partially transformed in slurries from the SR site.

The rate limiting step in this process is probably the initial transformation reaction of aniline and toluidine. Possible reactions include an aryl hydroxylation or the oxidation of the methyl group to form aminobenzoic acid. The anaerobic oxidation of an aryl methyl group with H_2O as a coreactant has been suggested previously for denitrifying bacteria degrading p-cresol or toluene (30, 31). Similar suggestions have been made for the degradation of p- and o-cresol under SRC (24, 32). An anaerobic aryl hydroxylation with H_2O has recently been demonstrated with benzene and toluene in a methanogenic consortium (33). To date, there has been no evidence for the catabolic pathways of aniline or toluidine in the absence of molecular oxygen.

Aminobenzoic acids can be anaerobically degraded under denitrifying (21), sulfate reducing (34; this study) and methanogenic conditions (35, 36; this study). The pathway of aminobenzoic acid decomposition is controversial. Decarboxylation and deaminations have been suggested as initial transformation mechanisms for such substrates (35). In addition, the formation of the CoA derivative of anthranilic acid was demonstrated in cell-free extracts (37). We did not find any potential metabolic intermediates of the aminobenzoic acids including benzoate, o-, m-, p-hydroxybenzoic acid, phenol or aniline in the aquifer slurries. We assume that decarboxylation was not the initial reaction in our experiments because we did not observe the accumulation of aniline in aquifer slurries amended with the aminobenzoate isomers. As already indicated, aniline persisted in aquifer slurries when it was used as a parent substrate. This is in contrast to benzoate and the hydroxybenzoates which were metabolized after only short lag periods (<2 weeks) at 1-2 order of magnitude higher rates (1.5-3.5 day^{-1}) than the aminobenzoates in samples from the same aquifer (38). Consequently, we would not expect the accumulation of such aromatic metabolites even if they were produced.

Evidence is accumulating that aryl amino substituents render the resulting aromatic compounds more resistent to anaerobic microbial metabolism. As indicated above, the anaerobic metabolism of hydroxy substituted benzoic acids proved much faster than the corresponding amino derivatives. The aryl amino and hydroxyl groups have similar mesomeric effects, but the reductive or hydrolytic cleavage of the amino substituent from the aromatic nucleus appears to be more difficult than the reductive removal of the hydroxy group. Generally, the removal of the carboxy group from p-hydroxybenzoate is relatively fast (38). However, whether aminobenzoate decarboxylation even occurs is unknown.

Similar effects can also be observed with anaerobic aryl dehalogenation reactions. Chloroanilines can be biotransformed by the reductive elimination of aryl halides (39), but the adaptation periods prior to the onset of this reaction exceed those typically observed for chlorophenols and chlorobenzoates by weeks. In contrast to the latter substrate groups, the polychloroanilines were converted to monohalogenated derivatives and were not completely mineralized in M aquifer slurries (39). Further, in experiments with a dehalogenating bacterial consortium, the rate of mono- and dihalobenzoate dehalogenation was reduced 89% and 63% respectively, by the introduction of a para amino group to parent substrate (40).

Benzeneamide and p-toluamide were relatively easily biodegraded in anaerobic samples from both aquifer sites. However, when the nitrogen atom was substituted with a single methyl group, the biodegradability of the respective N-methylbenzamide decreased in the M aquifer slurries. A second N-methyl group rendered the respective molecule highly persistent under both SRC and MC. Interestingly, N-methylbenzamide was degraded in the SR rather than M incubations. Conceivably, an N-demethylation reaction might be postulated and favored under the former conditions. N-dealkylation reactions are known degradation pathways for various pesticides including the s-triazines, phenylureas, dinitrotoluidine and the dipyridyl herbicides (41). However, most such studies were done under aerobic conditions and might involve the oxidation of the N-alkyl substituent prior to cleavage of the N-C bond (41). Whether such chemicals are metabolized through similar initial reaction sequences under aerobic and anaerobic conditions is unknown. In this context it is interesting to note that alkylamino hydrolase (dealkylaminase) activity was observed in

cell-free extracts of a *Pseudomonas* sp. strain under both aerobic and anaerobic conditions (42). The enzyme activity was detected during the degradation of N-cyclopropylmelamine.

Other reaction sequences must also be considered. For instance, Pereira and coworkers (43) found the anaerobic methylation (rather than demethylation) of nitrogen heterocyclic ground water contaminants. It is unclear whether the above reaction sequences or others are occurring in our experiments.

We are not aware of any reports describing the anaerobic metabolism of aryl sulfonates. In aquifer slurries from the SR and the M site only one of the model compounds, p-benzosulfonic acid, was biotransformed (Table 4). The chemical feature that this molecule possesses over others in its class is an aryl carboxy group. As with other classes of substrates (anilines, phenols), the presence of an aryl carboxy group makes the resulting molecule much more susceptible to anaerobic microbial attack. In general however, we must consider sulfonic acid derivatives as highly recalcitrant molecules in anoxic aquifers.

The carbon-sulfur bond of the sulfonate group attached to either an alkane or an aromatic ring is thermodynamically far more stable than the sulfate ester bond (C-O-S). This is reflected in the rarity of naturally occurring sulfonates and the increased biological recalcitrance of such compounds (15). To illustrate, alkylsulfatase activity (sulfate ester cleavage) could be found in 12 anaerobic bacteria including members of genera like *Clostridium*, *Lactobacillus*, *Peptococcus*, and *Eubacteria* (44). No mineralization of alkylsulfonates or alkylbenzenesulfonates could be detected in methanogenic sewage sludge incubations, whereas the same inoculum could anaerobically biodegrade dodecylsulfate (45). In the presence of molecular oxygen, the sulfonic group can be cleaved from the aromatic nucleus by the action of oxygenases (13, 46). In the absence of oxygen, this reaction cannot be employed, and it is not known if anaerobes have the potential to catabolize such compounds.

Benzenesulfonamides are generally more recalcitrant in anaerobic aquifer slurries if the molecule is alkylated at the N-atom. None of the model N-alkylated benzenesulfonamides were biotransformed in the anaerobic aquifer slurries, but benzenesulfonamide and p-toluenesulfonamide were degradable in M incubations. There is some evidence that the sulfonamide bond can be cleaved by microbes in the presence of molecular oxygen. Balba and coworkers (47) found sulfanilamide and sulfanilic acid in the culture fluid of an aerobic microbial consortium metabolizing the herbicide asulam (methyl(4-aminobenzenesulfonyl)carbamate). However, we were not able to detect benzenesulfonic acid in the aquifer slurries receiving benzenesulfonamide. The former structure is stable when incubated as parent substrate under the same conditions.

Interestingly, both benzamide and benzenesulfonamide were biotransformed when substituted with an aryl methyl group. However N-substitutions made these molecules more recalcitrant. That is, the benzamide derivative was less easily degraded when substituted with a single N-alkyl group whereas the corresponding benzenesulfonamide derivative proved persistent. Benzamide or benzenesulfonamide derivatives with two N-alkyl groups were not transformed at all. Apparently, the N-alkyl group of the benzamide derivative was less inhibitory to anaerobic biodegradation than that of the benzenesulfonamide. Conceivably, the degradation of these substrates might involve the initial hydrolysis of the amide bond. Consistent with our findings, an amidase from a *Brevibacterium* sp. was able to hydrolyze alkylamides and benzamides but not N-alkylamides (48). The same bacterium also possessed activity to hydrolyze nicotinamide.

The goal of this study was to examine the anaerobic biodegradation potential of a series of alkylated aromatic model compounds in anaerobic aquifer slurries. We conclude that most of the model aminosubstituted benzenes and alkylated benzamides can be biodegraded in SR or M aquifer slurries, but with high degree of variance in the rate of biotransformation. In contrast, most of the substituted sulfonated benzenes and benzenesulfonamides were highly recalcitrant under the same conditions. The development of environmental biorestoration strategies for the clean-up of contaminated environments requires reliable information on i) the susceptibility of contaminants to both aerobic and anaerobic destruction and ii) a knowledge of the predominant metabolic pathways. The latter point will allow a rational evaluation of the risks associated with this form of biotechnology. The study reported here represents an attempt to help provide a scientific basis for the consideration of anaerobic bioremediation scenarios. Since anaerobes can degrade substances that are recalcitrant under aerobic conditions, it is likely that anaerobic procedures will be increasingly important to the treatment of various wastes (49).

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